Metabolism, Pharmacokinetics, and Excretion of the 5-Hydroxytryptamine<sub>1B</sub> Receptor Antagonist Elzasonan in Humans

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ABSTRACT:

The metabolism, pharmacokinetics, and excretion of a potent and selective 5-hydroxytryptamine<sub>1B</sub> receptor antagonist elzasonan have been studied in six healthy male human subjects after oral administration of a single 10-mg dose of [<sup>14</sup>C]elzasonan. Total recovery of the administered dose was 79% with approximately 58 and 21% of the administered radioactive dose excreted in feces and urine, respectively. The average <i>t</i><sub>1/2</sub> for elzasonan was 31.5 h. Elzasonan was extensively metabolized, and excreta and plasma were analyzed using mass spectrometry and NMR spectroscopy to elucidate the structures of metabolites. The major component of drug-related material in the excreta was in the feces and was identified as 5-hydroxyelzasonan (M3), which accounted for approximately 34% of the administered dose. The major human circulating metabolite was identified as the novel cyclized indole metabolite (M6) and accounted for ~65% of the total radioactivity. A mechanism for the formation of M6 is proposed. Furthermore, metabolism-dependent covalent binding of drug-related material was observed upon incubation of [<sup>14</sup>C]elzasonan with liver microsomes, and data suggest that an indole iminium ion is involved. Overall, the major metabolic pathways of elzasonan were due to aromatic hydroxylation(s) of the benzylidene moiety, N-oxidation at the piperezine ring, N-demethylation, indirect glucuronidation, and oxidation, ring closure, and subsequent rearrangement to form M6.

Introduction

Selective serotonin reuptake inhibitors are thought to provide symptomatic relief from depression by blocking the reuptake of serotonin [5-hydroxytryptamine (5-HT)] in the synaptic cleft, thereby correcting deficits in 5-HT neurotransmission (Olvey and Skrepnek, 2008; Cashman et al., 2009). Similar to selective serotonin reuptake inhibitors, a 5-HT<sub>1B</sub> antagonist is also expected to elevate 5-HT tone but would do so by blocking the terminal autoreceptor that negatively modulates 5-HT release (Slassi, 2002). Elzasonan (4-(3,4-dichlorophenyl)-2-[2-(4-methyl-piperazin-1-yl)-benzylidene]-thiomorpholin-3-one; CP-448,187) exhibits potent and selective antagonism of 5-HT<sub>1B</sub> receptors in vitro, and preclinical in vivo studies demonstrate enhanced 5-HT neurotransmission.

The metabolism and pharmacokinetic of elzasonan were studied in animal species (Colizza et al., 2008; Kamel et al., 2009), and results indicated that most of the metabolic pathways were qualitatively similar to those of humans. However, the formation of the novel cyclized indole metabolite was detected at considerably lower levels in the plasma of preclinical species than in humans. There are no known circulating pharmacologically active metabolites of elzasonan, including the cyclized indole metabolite.

The present study was undertaken to assess the metabolic profile, the routes of excretion, and the pharmacokinetics of elzasonan in healthy male volunteers after a single dose of 10 mg of [<sup>14</sup>C]elzasonan. The metabolites of elzasonan were tentatively identified by ionspray LC-MS/MS using collision-induced dissociation (CID), neutral loss, total ion current, and precursor ion scanning techniques. NMR spectroscopy and chemical approaches were also used to identify the major human fecal and circulating metabolites. Furthermore, an investigation of a unique cyclized indole metabolite and its down

ABBREVIATIONS: 5-HT, 5-hydroxytryptamine, serotonin; CP-448,187, 4-(3,4-dichloro-phenyl)-2-[2-(4-methyl-piperazin-1-yl)-benzylidene]-thiomorpholin-3-one, elzasonan; LC, liquid chromatography; MS/MS, tandem mass spectrometry; CID, collision-induced dissociation; HPLC, high-pressure liquid chromatography; r, recombinant; CE-244,600, [4-(3,4-dichloro-phenyl)-2-[5-hydroxy-2-(4-methyl-piperazin-1-yl)-benzylidene]-thiomorpholin-3-one; CP-609,707, elzasonan N-oxide; CP-459,326, N-desmethylelzasonan; AUC, area under the plasma concentration-time curve; ARC, accurate radioactivity counting; P450, cytochrome P450.
stream iminium ion metabolite and role in in vitro covalent binding to liver microsomal protein is reported.

Materials and Methods

General Chemicals. Commercially obtained chemicals and solvents were of HPLC or analytical grade. Luna HPLC columns were obtained from Phenomenex (Torrance, CA). Ecolite(+) scintillation cocktail was obtained from MP Biochemicals (Solon, OH). Carbosorb and Permafluor scintillation cocktails were purchased from Packard Instrument Company (Downers Grove, IL). β-Glucuronidase from Helix pomatia (type H-1 with sulfatase activity) was obtained from Sigma-Aldrich (St. Louis, MO). Recombinant (r) CYP3A4 and CYP1A2 were purchased from BD Gentest (Woburn, MA).

Radiolabeled Drug and Reference Compounds. [14C]Elzasonan (free base, Fig. 1), labeled at the 2-position of the 2-(4-methyl-piperazin-1-yl)-benzylidene moiety attached to the thiomorpholin-3-one ring was synthesized by the radiochemistry group at Pfizer Global Research and Development (Groton, CT). [14C]Elzasonan had a specific activity of 15.66 mCi/mmol and a radiochemical purity of ≥99% as determined by HPLC with on-line radioactivity detection. 5-Hydroxyelzasonan ([4-(3,4-dichloro-phenyl)-2-(2-methyl-1,2,3,4-tetrahydropyridin-1-yl)-benzylidene]-thiomorpholin-3-one; CE-244.600), M6 [4-(3,4-dichlorophenyl)-2-(2-methyl-1,2,3,4-tetrahydroprazino[1,2-a]indol-10-yl)-thiomorpholin-3-one], elzasonan-N-oxide (CP-609,707), and N-desmethyelzasonan (CP-459,326) were synthesized by the Medicinal Chemistry group at Pfizer Global Research and Development and served as synthetic standards to confirm the structures. A detailed description of the synthesis of radiolabeled elzasonan and metabolite M6 are included in the supplemental data.

Urine, Feces, and Plasma Collection Methods. A mass balance and excretion study was conducted in six healthy male human subjects. Human subjects were administered a single 10-mg (free base) oral dose of elzasonan containing 100 μCi of [14C]elzasonan. Urine and feces were collected from the subjects in 24-h intervals for a minimum of 7 days to a maximum of 29 days postdose. The first urine sample was collected at 0 to 12 h postdose. All biological samples were stored at −20°C until analysis. Sample analysis was performed within days after the completion date of the study, and metabolite profiles on pooled samples were obtained.

For plasma profiles and metabolite identification, blood samples were collected into heparinized Vacutainers at the following time points: 2, 8, 24, 48, 96, and 192 h postdose. The blood samples were centrifuged at 4°C, and plasma was transferred to clean tubes. Plasma samples were pooled for each human subject and stored at −20°C until analysis.

Pooling Methods to Obtain Quantitative and Qualitative Data. It is essential that quantitative metabolite profiling represents accurate metabolite percentages relevant to the AUC (or Cmax) rather than an arbitrary plasma pool. Therefore, for quantitative profiling of metabolites in circulation, pooling was done according to the method whereby samples are pooled in proportion to the time interval each sample represents (Hamilton et al., 1981; Riad et al., 1991; Hop et al., 1998). Plasma samples were pooled such that at least 80% of the total radiolabel AUC was represented. For subsequent metabolite identification, the most concentrated samples were used to avoid dilution of circulating drug-related materials.

For quantitative profiling of metabolites in excreta (urine and feces), samples were pooled from each individual in proportion to the amount (weight or volume) of excreta in each sampling period. For subsequent metabolite identification (qualitative analysis), the most concentrated samples were used to avoid unnecessary dilution of metabolites.

Determination of Total Radioactivity. Total radioactivity in urine and plasma was determined by counting sample aliquots (50–100 μl) using a 4°C program on a Wallac 1409 liquid scintillation counter (PerkinElmer Life and Analytical Sciences, Waltham, MA). Ecolite(+) scintillation cocktail (20 ml) was used for the determination of the radioactivity in the samples. Quench curves were prepared using [14C]elzasonan at different concentrations.

Fecal samples at each time point were suspended in 50 to 200 ml of water based on sample weight and homogenized, and the total weights of the fecal homogenate were recorded. Aliquots (90–115 mg, in triplicate) of the homogenates were air-dried and combusted in a Packard TriCarb Oxidizer (PerkinElmer Life and Analytical Sciences). The liberated 14CO2 was trapped in Carbosorb and Permafluor scintillation cocktails (15 ml; PerkinElmer Life and Analytical Sciences), and the radioactivity in the trapped samples was determined by counting on a liquid scintillation counter. Combustion efficiencies were determined by combusting 14C standards (Spec-Chec; PerkinElmer Life and Analytical Sciences) in an identical manner. The samples obtained at predose were used as controls and counted to obtain a background count rate. The radioactivity in urine and feces at each sampling time was expressed as the percentage of dose excreted in the respective matrices at that sampling time.

Concentrations of Total Radioactivity and Elzasonan. Blood samples (10 ml) from each subject (n = 6) were collected into heparinized Vacutainers at the following time points: 0, 2, 3, 4, 6, 8, 12, 24, 48, 72, 96, 144, 240, 288, 336, 384, 432, 480, 504, and 552 h postdose and were centrifuged. The resulting plasma samples were placed into clean tubes for shipment and subsequent analysis. Total radioactivity in plasma was determined by counting 500-μl aliquots from each sample in duplicate using a 14C program on a Wallac 1409 liquid scintillation counter. Ecolite(+) scintillation cocktail (~18.0 ml) was used for the determination of radioactivity in the samples. Total radioactivity in the plasma samples was converted into nanogram equivalents per milliliter based on the specific activity (the amount of radioactivity per unit amount of substance) received by each patient (100 μCi of 14C/10 mg of elzasonan). Plasma concentrations of the unchanged elzasonan (nanograms per milliliter) were determined by a validated HPLC-MS/MS assay (Northwest Bioanalytical, Salt Lake City, Utah).

In Vivo Covalent Binding Experiments. [14C]Elzasonan (10 μM; 58 mCi/mmol) was incubated with pooled human, female rat, and dog liver microsomes (1.0 mg/ml protein) in a total volume of 0.8 ml of KH2PO4 (100 mM, pH 7.4) containing 3.3 mM MgCl2. Incubations were commenced with the addition of NADPH to a final concentration of 1.3 mM. Each determination was made in triplicate. In human liver microsomal incubations, the effect of various model nucleophiles and other factors were examined: KCN (0.1 mM), GSH (5 mM), lysine (1 mM), ketoconazole (1.0 μM), or human liver cytosolic fraction (3 mg/ml). Incubations were conducted for 20 min at 37°C in a shaking water bath open to the air. Reactions were terminated by the addition of 5 volumes of CH3CN (4 ml) to precipitate the microsomal protein. The terminated incubation mixture was vortex-mixed and centrifuged for 5 min (Jouan swinging bucket, 2000 rpm). The supernatant was removed, and the protein pellet was washed four times with 4.8 ml of H2O-CH3CN (20:80) and five times with 4 ml of CH3CN. Washes consisted of vortex mixing of the pellet in a tube containing metal wire to assist in disruption of the pellet, followed by centrifugation as above. Washing continued until there remained no more detectable radioactivity in the supernatant. The final pellet was dissolved in 1 ml of 2 M NaOH, and the resulting solution was analyzed by liquid scintillation counting in 18 ml of True-Count. To test the acid liability of the covalently bound material, one determination was made by initially precipitating the pellet with 0.1 M HClO4.

The biosynthesized [14C]elzasonan indole iminium ion metabolite (see below) was also examined for in vitro covalent binding to pooled human liver microsomes. The [14C]iminium ion (0.18 μM) was incubated with pooled human liver microsomes (1.0 mg/ml) in 0.8 ml of KH2PO4 (100 mM, pH 7.4) containing 3.3 mM MgCl2. Incubations were conducted in the presence or absence of NADPH (1.3 mM), KCN (1.0 mM), or GSH (10 mM). Incubations were conducted for 20 min at 37°C in a shaking water bath open to the air. Reactions were terminated by the addition of 4 ml of CH3CN, and protein pellets were washed and analyzed as described above.
Biosynthesis of the Elzasonan Indole Iminium Ion Metabolite. The iminium ion metabolite was biosynthesized using recombinant heterologously expressed human CYP3A4. Elzasonan (20 μM) was incubated with rCYP3A4 (1 mg/ml protein) and NADPH (1.3 mM) in 2 ml of KH₂PO₄ (pH 7.45, 100 mM) and 3.3 mM MgCl₂. The incubation was conducted at 37°C in a shaking water bath open to the air for 30 min. The reaction was terminated by the addition of 6 ml of CH₃CN, and the precipitated protein was removed by centrifugation. The supernatant was evaporated in a TurboVap at room temperature, and the residue was reconstituted in 10 mM NH₄OAc containing 5% CH₃OH at a flow rate of 0.8 ml/min. This condition was held for 5 min followed by a linear gradient to 80% CH₃OH over 20 min rated in a TurboVap at room temperature, and the residue was reconstituted in radioactivity was recovered in the supernatants. The supernatant was concentrated and reconstituted in 200 ml of 10 mM ammonium acetate (pH 4.0)/methanol-dimethyl sulfoxide (1.5:1.5:1). The iminium ion was purified by HPLC. The sample was injected onto a Luna C18 column (4.6 × 150 mm, 5 μm; Phenomenex) equilibrated in 10 mM NH₄OAc containing 5% CH₃OH at a flow rate of 0.8 ml/min. This condition was held for 5 min followed by a linear gradient to 80% CH₃OH over 20 min and holding at this solvent composition for 5 min. Fractions were collected each minute. The effluent was monitored with UV detection at 353 nm. The fraction eluting at 23.5 min was analyzed by direct injection into a Sciex API100 mass spectrometer and identified as the iminium ion metabolite. The fraction was stored in the freezer in an amber vial. Re-injection of this fraction on a HPLC-MS system the following day demonstrated that the iminium ion was stable. A sample of [¹⁴C]iminium ion was biosynthesized and purified in the same manner, using [¹⁴C]elzasonan as the substrate. The molecular ion of the [¹⁴C]iminium ion was m/z 446, consistent with the enrichment of one ¹⁴C atom.

Pharmacokinetic Analysis. Pharmacokinetic parameters were determined using the WinNonlin noncompartmental analysis computer program (version 3.2, Pharsight, Mountain View, CA). The areas under the elzasonan and total radioactivity AUCs were calculated from plasma concentrations of elzasonan and total radioactivity using a linear trapezoidal approximation of area with zero as the time 0 concentration. The maximum observed plasma concentration (Cmax) was estimated directly from experimental data, and time to maximum plasma concentration (Tmax) was defined as the time of first occurrence of Cmax. The terminal elimination phase rate constant (Kt) was estimated using least-squares regression analysis of the plasma elzasonan concentration-time data obtained during the terminal log-linear phase. The terminal phase half-life (t1/2) was calculated as ln2/Kt, whereas the mean t1/2 was estimated as ln2/mean Kt.

Extraction of Metabolites from Biological Samples. Urine. Urine samples were pooled by subject as described above. The pooled samples were vortex-mixed, and 5 ml from each pool was evaporated to dryness in a TurboVap. The residue was reconstituted in 200 μl of 10 mM ammonium acetate (pH 4.0)-methanol-dimethyl sulfoxide (1.5:1.5:1). Aliquots (100 μl) were directly injected without further purification onto the LC-accurate radioisotope counting (ARC) system (described below) for metabolite profiling. For metabolite identification, approximately 80-ml urine pools were concentrated and dried in a TurboVap at room temperature and then reconstituted in 600 μl of 10 mM ammonium acetate (pH 4.0)-methanol-dimethyl sulfoxide (1.5:1.5:1). Aliquots (90 μl) were injected onto the HPLC-MS/MS system. The LC system is described below.

Feaces. Fecal samples were pooled according to sample weight as described above. For metabolite profiling, approximately 1 g of each sample pool was extracted with 7 ml of acetonitrile-H₂O (6.1 v/v) twice; 100-μl aliquots of both extractions were counted to determine recovery. Approximately 87% of the radioactivity was recovered in the supernatants. The supernatant was evaporated in a TurboVap at room temperature, and the residue was reconstituted in 500 μl of 10 mM ammonium acetate (pH 5.0)-methanol-dimethyl sulfoxide (1.5:1.5:1). Aliquots (100 μl) were injected onto the LC-ARC system for profiling. For metabolite identification, 10 g of each sample pool was extracted with 30 ml of acetonitrile-H₂O (5:1 v/v) twice. The supernatants were combined and dried in a TurboVap at room temperature. The dried residue was dissolved in 5 ml of water and extracted with 15 ml of hexane twice. The hexane layer was removed, and the aqueous layer was further extracted with 15 ml of ethyl acetate twice. The extractions were combined and dried in the TurboVap at room temperature, and the residue was reconstituted in 360 μl of 10 mM ammonium acetate (pH 6.0)-methanol-dimethyl sulfoxide (1.5:1.5:1). Aliquots of 90 μl were injected onto the HPLC-MS/MS system for metabolite identification.

Plasma. Plasma samples were pooled by human subject separately according to the method described above. Aliquots (1 ml) from each pool were extracted with 5 ml of acetonitrile. The mixtures were vortex-mixed for 3 min, sonicated for 2 min, and then centrifuged for 5 min at 20°C. Aliquots of the supernatant (two 500-μl aliquots) were counted to determine recovery. Approximately 95% of the radioactivity was recovered in the supernatants. The supernatants were combined according to subject number to two equal sets and dried in the TurboVap at room temperature. One set of the dried residue (six subjects) was reconstituted in 150 μl of 10 mM ammonium acetate (pH 4.0)-methanol-dimethyl sulfoxide (1.5:1.5:1), and aliquots of 100 μl were injected onto LC-ARC system for profiling. The other sets of dried residues were combined and reconstituted in 300 μl of 10 mM ammonium acetate (pH 4.0)/methanol-dimethyl sulfoxide (1.5:1.5:1), and 90 μl was injected onto HPLC-MS/MS system for metabolite identification.

Quantitative Assessment of Metabolites Using Radiometric Detecting Method. Quantification of the metabolites was performed by measuring radioactivity in the individual HPLC-separated peaks using a β-RAM instrument (IN/US Systems, Tampa, FL). The β-RAM provided an integrated display in counts per minute and the percentage of the radiolabeled material as well as peak representation. The β-RAM was operated in the homogeneous liquid scintillation counting mode with addition of 4 ml/min of Ecolliter (+) scintillation cocktail to the eluent after UV detection. Urinary, fecal, and plasma profiles were quantitatively very similar over time for at least 12 months, indicating sample stability in these matrices.

Enzyme Hydrolysis. Pooled human urine samples (~10 ml) were adjusted to pH 5 with sodium acetate buffer (0.1 M) and treated with 2500 units of β-glucuronidase/sulfatase. The mixture was incubated in a shaking water bath at 37°C for 12 h and was diluted with acetonitrile (~10 ml). The precipitated protein was removed by centrifugation. The pellet was washed with an additional 2 ml of acetonitrile, and both supernatants were combined. The supernatant was concentrated and dissolved in 0.5 ml of mobile phase, and an aliquot (50 μl) was injected on the HPLC column. Incubation of the urine samples without the enzyme served as a control.

Reduction of N-Oxide Metabolite with TiCl₄. The extracted human plasma samples or the dried human urine samples were reconstituted in 100 μl of 10 mM ammonium acetate (pH 4.0)-methanol-dimethyl sulfoxide (1.5:1.5:1). Titanium chloride solution (titanium chloride-20% hydrochloric acid; Thermo Fisher Scientific, Waltham, MA) was added drop by drop until the pink color of the reaction mixture persists. Then 100 μl of the reaction mixture was injected onto the LC-MS system.

Sample Preparation for ¹H NMR Analysis of the Major Human Fecal and Plasma Metabolites: Major Human Fecal Hydroxylated Metabolite M3. Ten-gram aliquots of pooled human feces were extracted twice with 70 ml of water-acetone-trit (1:6: v/v). This process was repeated for a total of eight extractions to ensure adequate material for spectroscopic characterization. The supernatants were dried in a TurboVap. The dried samples were redissolved in 5 ml of water, extracted with 5 ml of hexane twice (hexane extracts were discarded), and then extracted with 5 ml of ethyl acid twice. The ethyl acid extracts were combined and dried in a TurboVap and supernatant was concentrated and dissolved in 0.5 ml of mobile phase, and an aliquot (50 μl) was injected on the HPLC column. Incubation of the urine samples without the enzyme served as a control.

HPLC Purification of M3. Normal-phase HPLC was used for the purification of M3. Chromatography was performed on a Zorbax Sili HPLC column (4.6 × 150 mm, 5 μm) with a binary mixture of hexane (solvent A) and ethanol (solvent B). A flow rate of 1 ml/min was used for the isolation and purification of M3. The mobile phase initially consisted of solvent A-solvent B (75:25, v/v) for 15 min. It was then linearly programmed to 100% over a 1-min period. The system was operated isocratically for 4 min and then linearly programmed back to the initial condition in 1 min and equilibrated for 4 min. The dried ethyl acid extracts residue was loaded into the HPLC-β-RAM system to determine the retention time of M3 under the HPLC condition described above. Injections were then loaded into the HPLC/CUV system (LDC Analytical; Thermo Instrument Systems Inc., Santa Fe, NM), and M3 fractions were collected, combined, and dried in a TurboVap and finally frozen at −20°C for ¹H NMR analysis.

Large-Scale Biosynthesis of Major Human Circulating Metabolite (M6). In vitro experiments using various subcellular fractions as well as recombinant P450 to generate M6 in enough quantity for NMR analysis were unsuccessful. Therefore, a unique approach was taken to generate M6 via chemical reduction of the biosynthesized iminium ion metabolite. The reaction mixture consisted of 900 μl of stock [¹⁴C]elzasonan (20 μM) (1 μg/μl in 50:50 MeOH-H₂O), 3600 μl of rCYP3A4 (9.6 mg of protein/ml, 109 pmol of
Metabolism and Disposition of Elzasonan

P450 (mg of protein, final concentration 37 nM), 7550 μl of rCYP1A2 (8.6 mg of protein/ml, 38.4 pmol of P450/mg of protein, final concentration 25 nM), and 7795 ml of phosphate buffer (pH 7.4). The reaction was initiated with 10 ml of cofactor solution (40 mg of β-NADP, 160 mg triisocitric acid + 9.785 ml of 125 mM MgCl2 + 215 μl of isocitric acid dehydrogenase). This gave a final incubation volume of 100 ml. The incubation was performed in 37°C for 1 h. For NMR analysis, the same set of experiments was performed using unlabeled elzasonan. After incubation, the reaction mixture was divided equally into 50 tubes (2.0 ml in each tube) and stopped by the addition of 5.0 ml of methyl tert-butyl ether to each tube. Each tube was vortex-mixed using a Multivortex mixer, centrifuged for 6 min, and then flash frozen on methanol with dry ice. The supernatants were combined and dried in a TurboVap.

Quantitative assessment of the reduction of elzasonan-N-oxide to elzasonan suggested that CYP1A2 may be responsible for this reductive pathway (Kamel et al., 2009). Therefore, CYP1A2 was added to the incubation mixture to block or minimize the oxidative pathway for the formation of the major N-oxide metabolite and thus drive the reaction to increase the yield for the formation of the indole iminium ion metabolite. This process proved to be successful to obtain a much larger quantity of elzasonan indole iminium ion metabolite.

NaBH₄ Reduction. Elzasonan indole iminium ion metabolite was isolated and purified from the large-scale incubated sample using HPLC. The dried indole iminium ion sample was reconstituted in 200 μl of anhydrous methanol and approximately 20 μg of NaBH₄ (98%; Aldrich Chemical Company, Milwaukee, WI) was added. The reaction was performed on ice for 30 min and dried in the TurboVap. The same experiment was performed for unlabeled elzasonan.

HPLC Purification of the Reduced Indole Iminium Ion Metabolite. The NaBH₄ reduced sample was reconstituted in 800 μl of 10 mM NH₄OAc (pH 5)-MeOH-H₂O at the ratio of 1.5:1:5:1:0. Multiple 100-μl aliquots were then injected onto an HPLC system that consisted of a HP-1050 autosampler and a HP-1050 gradient pump. Chromatography was performed on a Luna HPLC column [4.6 × 150 mm, 5 μm, C18(2)]; Phenomenex] using a binary mixture of 10 mM ammonium acetate, pH 5, with acetic acid (solvent A) and 50:50 acetonitrile-CH₃OH (solvent B). A flow rate of 1.0 ml/min was used. The mobile phase initially consisted of solvent A-solvent B (60:40) for a 4-min period. It was then linearly programmed to achieve a mixture of solvent A-solvent B (30:70) over a 15-min period. The system was operated isocratically for another 3 min and then linearly programmed to achieve a mixture of solvent A-solvent B (10:90) over a 3-min period. The system was operated isocratically for another 3 min and then returned to the initial mixture of solvent A-solvent B (60:40) over a 2-min period and remained for another 10 min. This gave a total run time of 40 min. The fraction at retention time 24.2 to 25.2 min where M6 was eluted was collected, combined, dried in a TurboVap, and stored at −20°C for LC-NMR analysis.

pH Titration Curve and Reaction of the Iminium Ion Metabolite with Cyanide and GSH. The indole metabolite (20 μM) was incubated with male monkey liver microsomes (2 mg/ml), NADPH (1.3 mM), and MgCl₂ (3.3 mM) in 20 ml of KH₂PO₄ at 37°C for 40 min to biosynthesize the iminium ion metabolite. The incubation was terminated with CH₃CN (20 ml) and spun in a centrifuge at 1700g. To the supernatant was added 100 ml of 0.1% HCOOH, and the solution was spun in a centrifuge at 40,000g. The entire supernatant was applied to a Polaris C18 column (4.6 × 250 mm; 5 μm) at a flow rate of 0.8 ml/min. The iminium ion metabolite was eluted using a gradient beginning with 0.1% HCOOH in 30% CH₃CN, which was held for 5 min followed by a linear increase in CH₃CN to 60% at 50 min. The fractions eluting between 20.0 and 21.5 min contained the iminium ion and were pooled and used in subsequent experiments.

Because of its conjugated nature, the iminium ion metabolite possesses a strong absorbance at 360 nm. To determine the pH at which the iminium is converted to a carboxilamine, a portion of the isolated fraction was examined for absorbance between 500 and 200 nm while the pH was increased with K₂HPO₄. Reactions between the iminium ion and cyanide ion and with reduced GSH were examined in solutions containing 0.5 M KH₂PO₄ at pH 7.5 by monitoring the absorbance spectrum.

HPLC/ARC for the Separation of All Plasma Metabolites. HPLC-ARC provides several advantages over traditional detection systems (Nassar et al., 2003; Wang et al., 2009) and was used for plasma profiling. The HPLC-ARC system consisted of an HP 1100 binary pump with an Agilent 1100 autosampler, an ARC stop-flow system (AIA Research Co., Newark, DE), and a β-RAM detector. The flow cell (2.2 ml volume) and cocktail were obtained from AIA Research Co. The flow was stopped in 10-s intervals and counted for 60 s. The 10 s of flow (−0.17 ml) mixes with enough scintillation cocktail to fill the cell for any given stop-flow period. The background counting threshold was 30 cpm. Chromatography was identical to that used for the purification of the reduced iminium ion metabolite as described above.

Mass Spectrometry. Analysis of metabolites was conducted with a SCIEX API III™ (Perkin-Elmer Life and Analytical Sciences, Thornhill, ON, Canada) equipped with an ionspray source. The effluent from the HPLC column was split, and approximately 50 μl/min was introduced into the atmospheric pressure ionization source. The remaining effluent was directed into the flow cell of the β-RAM detector. The β-RAM response was recorded in real time by the mass spectrometer data system that provided simultaneous detection of radioactivity and mass spectrometry data. The delay in response between the two detectors was approximately 0.2 min with the mass spectrometric response recorded earlier. The ionspray interface was operated at 5000 V, and the mass spectrometer was operated in the positive mode. CID studies were performed using argon gas at a collision energy of 25 to 28 eV and a collision gas thickness of 3.5 × 10⁻¹⁴ molecules/cm².

NMR. Samples were analyzed by an LC-MS-NMR system consisting of an Agilent 1100 binary pump (Agilent Technologies, Santa Clara, CA), a Bruker BioSpin BPSU-0 column oven (Bruker BioSpin Corporation, Billerica, MA), a Bruker BioSpin photodiode array detector, a Bruker BioSpin BNMI interface using a 20:1 split, a Bruker Daltonics Enquire 3000 ion trap mass spectrometer (Bruker Daltonics, Billerica, MA) equipped with an electrospray source, a Bruker BioSpin BPSU-36 peak storage unit, and a Bruker 500 MHz Avance DRX spectrometer equipped with a 4-mm H⁻¹⁻¹C inverse-g gradient LC flow probe. Analyte within a sample aliquot (100 μl) was eluted at 0.5 ml/min over a reverse-phase column (5 μm, 3 × 150 mm) at room temperature using an aqueous buffer of (10 mM CD₃COOD in D₂O adjusted to pH 4 with ND₃OD) or (0.1% CF₃COOD in D₂O) and using CD₃OD or CD₃CN as organic eluent. Five percent of the column effluent was split postcolumn and diluted with 10% D₂O in CD₃CN (containing 0.2% CD₃COOD) at a flow rate of 125 μl/min before entering the mass spectrometer; the remaining 95% of the effluent passed through the diode array detector. The peaks of interest were stored in the BPSU-36 unit using the loop storage technique and subsequently introduced into the NMR spectrometer. ¹H and correlation spectroscopy spectra were obtained on LC peaks of interest using double presaturation of solvent NMR resonances. Proton chemical shifts are reported in parts per million relative to tetramethylsilane as referenced from the shift of residual protons in CD₃OD at 3.33 ppm.

Results

Excretion. The mean percentage of total radioactivity recovered from 0 to 29 days in urine and feces of six human subjects after oral administration of [¹⁴C]elzasonan is shown in Table 1. The average cumulative recovery in human urine, feces, and the sum of urine and feces is depicted in Fig. 2.

Pharmacokinetics. The mean plasma concentration-time curve for elzasonan (nanograms per milliliter) and total circulating radioacti-
vity (nanogram equivalents per milliliter) are graphically depicted in Fig. 3. A comparison of the $\text{AUC}_{0-\text{t} \text{last}}$, $t_{1/2}$, and $C_{\text{max}}$ between drug and total radioactivity is shown in Table 2.

**Metabolic Profiles in Urine, Feces, and Plasma.** Urine, fecal, and plasma profiles obtained for all subjects were of similar quality. Representative HPLC radiochromatograms of metabolites in human urine (0–96 h), feces (24–216 h), and plasma (0–96 h) after oral administration of $[^{14}C]$elzasonan are depicted in Fig. 4. The urine profiles for all subjects were qualitatively similar. Qualitative similarities were also observed for all fecal profiles. However, there were some quantitative differences in the excretion of metabolites in urine and feces. Qualitative and quantitative similarities of the circulating metabolites were observed for all human subjects. A summary of the abundance and description of the major human metabolites in urine, feces, and plasma is presented in Table 3. In addition to the parent drug, a total of two metabolites in urine, four metabolites in feces, and three metabolites in plasma were identified. M3, M5, and M6 were the major metabolites identified in feces, urine, and plasma, respectively.

**Identification of Metabolites.** The structures of metabolites were elucidated by ionspray LC-MS/MS, NMR, and LC-NMR, as well as chemical approaches or comparison to synthetic standards, when available. Combined liquid chromatography-ionspray mass spectrometry (full scan) and tandem mass spectrometry, such as precursor ion, product ion, single ion monitoring, and multiple reaction monitoring scanning techniques were used for the identification of metabolites. Where possible, the identities of metabolites were confirmed by coelution on HPLC with synthetic standards. The word “tentative” was used when the exact sites of some structural modification could not be determined.

Elzasonan had a retention time of $\sim 27.8$ min on the HPLC system and showed a protonated molecular ion at $m/z$ 448. The CID mass spectrum of elzasonan (Fig. 5) showed fragment ions at $m/z$ 433, 391, and 348, which represent the dichloro-phenylbenzylidene-thiomorpholinone moiety. The fragment ions at $m/z$ 228, 199, and 185 represent the piperazinyl-benzylidene moiety. The fragment ions at $m/z$ 204 and 144 represent the benzylidene moiety.
The fragment ions at $m/z$ 72 and 70 were the most abundant ions in the elzasonan mass spectrum and were derived from the methylpiperazine moiety.

Parent drug and a total of six metabolites were identified. Unchanged drug was found in urine, feces, and plasma samples. The structures of these metabolites were identical to those identified previously in preclinical species (Colizza et al., 2008; Kamel et al., 2009). M6 was a novel major circulating metabolite and required further structural confirmation.

**M1.** M1 had a retention time of 21.6 min on the HPLC-ARC system and showed a protonated molecular ion at $m/z$ 640, 192 mass units higher than that of the unchanged drug ($m/z$ 448), suggesting that it was a conjugate. M1 was only detected in urine and accounted for ~5.1% of the dose (average of six human subjects). Its CID spectrum (data not shown) showed the fragment ion at $m/z$ 464, a loss of 176 mass units, suggesting that M1 was a glucuronide conjugate. The fragment ion at $m/z$ 464, 16 mass units higher than the molecular ion of unchanged drug, suggested the addition of an oxygen atom to the molecule. The absence of water loss (18 amu) in the CID spectrum of M1 suggested that aliphatic hydroxylation had not occurred. Further collision-induced dissociation of the fragment ion at $m/z$ 464 (data not shown) showed the fragment ion at $m/z$ 215, indicating that the oxidation had occurred remote from the dichloro-phenyl-thiomorpholinone moiety. The fragment ion at $m/z$ 215, 16 Da higher than the fragment ion at $m/z$ 199 of the unchanged drug, further suggested that the benzylidene moiety was the site of oxidation. The exact position of the hydroxylation was determined from NMR data.

**M3.** M3 had a retention time of ~25.9 min on the HPLC-ARC system and showed a protonated molecular ion at $m/z$ 464, 16 mass units higher than that of the parent drug, suggesting that the molecule had undergone monooxidation. M3 accounted for 34.4% of the administered dose (average of six human subjects) in the fecal samples and was not detected in urine or plasma. Its CID spectrum is depicted in Fig. 6 and showed the fragment ion at $m/z$ 449, loss of 15 mass units ($-\text{CH}_3$), suggesting that no modification had occurred on the piperazine ring. The fragment ions at $m/z$ 72, 70, and 58 further suggested that the piperazine ring was intact. The fragment ions at $m/z$ 244, 215, and 190 indicated that the oxidation had occurred remote from the dichloro-phenyl-thiomorpholinone moiety. The fragment ions at $m/z$ 160 and 146, 16 Da higher than the fragment ions at $m/z$ 144 and 130 of the unchanged drug, suggested that the benzylidene moiety was the site of oxidation. The fragment ion at $m/z$ 201, 16 Da higher than the fragment ion at $m/z$ 185 of the unchanged drug, further suggested that the benzylidene moiety was the site of oxidation. The exact position of the hydroxylation was determined from NMR data.

Figure 7 shows a comparison of the aromatic region of the $^1H$ spectrum for the purified fecal hydroxylated metabolite M3 (top) and elzasonan (bottom). The $^1H$ spectrum for M3 shows a change in the aromatic region, consistent with hydroxylation at either position 5 or 6. The signals at 7.62 (d), 7.34 (d), and 7.67 (d) indicate that the dichloro-phenyl moiety is unchanged. The singlet at 7.99 indicates that the olefinic proton is unchanged. The remaining aromatic resonances 7.12 (d), 6.89 (dd), and 7.28 (d) are consistent with hydroxylation at position 5. However, based on the metabolite $^1H$ spectrum alone, it is not possible to definitively rule out hydroxylation at position 4. Comparison of the metabolite $^1H$ spectrum with a spectrum of a genuine synthetic standard CE-244,600 (data not shown) further supported this structure assignment and indicated that the two compounds have the same spectral pattern and therefore the same chem-structure. M3 was coeluted with the synthetic standard on HPLC and had an identical CID mass spectrum (Fig. 6). Based on these data, M3 was unequivocally identified as 4-(3,4-dichloro-phenyl)-2-(5-hydroxy-2-(4-methyl-piperazin-1-yl)-benzylidene)-thiomorpholin-3-one.

**Unchanged drug (elzasonan).** Elzasonan had a retention time of ~27.8 min on the HPLC-ARC system and showed a protonated molecular ion at $m/z$ 448. Parent drug accounted for ~5.5% of the administered dose in fecal samples and ~5% in urine. Unchanged drug coeluted with synthetic standard on the HPLC system and had an identical CID spectrum.

**M4.** M4 had a retention time of ~27.9 min on the LC-ARC system and showed a protonated molecular ion at $m/z$ 434, 14 mass units lower than the drug, suggesting that the molecule had undergone N-demethylation. M4 accounted for ~3.8% of the total radioactivity (average of six human subjects) in plasma and was not detected in urine or feces. M4 was coeluted with the synthetic standard and had an identical CID mass spectrum (data not shown). Based on these data, M4 was identified as 4-(3,4-dichlorophenyl)-2-(2-piperazin-1-yl-benzylidene)-thiomorpholin-3-one.

**M5.** M5 had a retention time of ~30.5 min on the HPLC-ARC system and showed a protonated molecular ion of $m/z$ 464, 16 mass units higher than that of the parent drug, suggesting that the molecule had undergone monooxidation. M5 accounted for ~5.6% ($M5 + M6$) of the administered dose in fecal samples, ~7.8% in urine, and ~5.5% of the total radioactivity in plasma (average of six human subjects). The CID mass spectrum of M5 (data not shown) showed the fragment ion at $m/z$ 447, a loss of 17 Da from $m/z$ 464, suggesting that the molecule had undergone N-oxidation. The absence of a water loss (18 mass units) in the CID spectrum of M5 further suggested that aliphatic hydroxylation had not occurred. Treatment of human urine and plasma metabolites with aqueous TiCl₃ resulted in the disappear-
ance of M5 and an increase of the response of unchanged drug in the radiochromatogram (data not shown). The CID mass spectra of M5 from plasma and urine were also identical to that of the synthetic standard. Based on these data, M5 was identified as elzasonan N-oxide.

**Indole iminium ion metabolite.** The indole iminium metabolite was not detected in humans and showed a molecular ion at m/z 444, 4 Da lower than that of the parent drug. Its CID spectrum (data not shown) showed the major fragment ions at m/z 225 and 229 and suggested that the parent drug had undergone oxidation followed by cyclization. The fragment ion at m/z 225, 3 mass units lower than the parent drug, suggested that the nitrogen is charged and the observed molecular ion at m/z 444 is M⁺, not [M + H]⁺. Figure 8 shows a comparison of the ¹H spectrum of elzasonan (top) and the indole iminium metabolite (bottom). By inspection, it is clear that there has been a major change to the metabolite. There is a shift in the N-methyl resonance from 2.82 ppm in the elzasonan spectrum to 3.69 ppm in the indole iminium metabolite spectrum, indicative of a positive charge and consistent with a quaternary nitrogen. The correlation spectroscopy spectrum (data not shown) shows a correlation from this methyl resonance at 3.69 ppm to a new significantly downfield singlet at 9.02 ppm as well as to a methylene resonance at 4.15 ppm. In addition, the singlet at 7.79 ppm in the elzasonan spectrum is missing in the indole iminium metabolite spectrum, and there is a new singlet at 5.68 ppm. In addition, there is a significant change in the overall UV profile (data not shown) with a shift in the UV maxima from 331 nm in the parent spectrum to 358 nm in the metabolite spectrum, consistent with a more conjugated chromophore in the metabolite. These observations can only be explained by the formation of an indole iminium ion.

**M6.** M6 coeluted with M5 and had a retention time of 30.5 min on the LC-ARC system. M6 showed a protonated molecular ion at m/z 200 ppm in the elzasonan spectrum to 3.69 ppm in the indole iminium metabolite spectrum, indicative of a positive charge and consistent with a quaternary nitrogen. The correlation spectroscopy spectrum (data not shown) shows a correlation from this methyl resonance at 3.69 ppm to a new significantly downfield singlet at 9.02 ppm as well as to a methylene resonance at 4.15 ppm. In addition, the singlet at 7.79 ppm in the elzasonan spectrum is missing in the indole iminium metabolite spectrum, and there is a new singlet at 5.68 ppm. In addition, there is a significant change in the overall UV profile (data not shown) with a shift in the UV maxima from 331 nm in the parent spectrum to 358 nm in the metabolite spectrum, consistent with a more conjugated chromophore in the metabolite. These observations can only be explained by the formation of an indole iminium ion.

![Figure 4](image-url)

**Fig. 4.** Representative HPLC radiochromatograms of urinary (subject 2, 0–96 h postdose pooled), fecal (subject 6, 24–216 h postdose pooled), and plasma (subject 5, 0–96 h postdose pooled) metabolites of elzasonan after a single oral administration of [¹⁴C]elzasonan at a dose of 10 mg.

**TABLE 3**

Average percentages of urinary, fecal, and plasma metabolites of elzasonan in human subjects after administration of a 10-mg (free dose) oral dose

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>m/z</th>
<th>Description</th>
<th>% Dose* Urine</th>
<th>% Total Radioactivity in Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>640</td>
<td>Hydroxyglucuronide</td>
<td>5.1 ± 3</td>
<td>N.D.</td>
</tr>
<tr>
<td>M2</td>
<td>464</td>
<td>3-, 4-, or 6-OH**</td>
<td>N.D.</td>
<td>6.2 ± 2</td>
</tr>
<tr>
<td>M3</td>
<td>464</td>
<td>5-OH</td>
<td>N.D.</td>
<td>34.4 ± 6</td>
</tr>
<tr>
<td>Parent</td>
<td>448</td>
<td>Elzasonan</td>
<td>5.0 ± 2</td>
<td>5.5 ± 1</td>
</tr>
<tr>
<td>M4</td>
<td>434</td>
<td>N-Desmethyl</td>
<td>N.D.</td>
<td>20.2 ± 4</td>
</tr>
<tr>
<td>M5</td>
<td>464</td>
<td>N-Oxide</td>
<td>7.8 ± 2</td>
<td>5.6 ± 1 (M5 + M6)</td>
</tr>
<tr>
<td>M6</td>
<td>446</td>
<td>Cyclized indole product</td>
<td>N.D.</td>
<td>65.2 ± 6</td>
</tr>
<tr>
<td>Totals</td>
<td></td>
<td></td>
<td>69.6</td>
<td>94.7</td>
</tr>
</tbody>
</table>

N.D., not detected; OH, hydroxy.

*Calculated on the basis of total %¹⁴C excreted from urine and feces of each subject shown in Table 1 (dose metabolite number = % radioactivity metabolite number × total %¹⁴C excreted).

**Most likely position 3 on the basis of electronic consideration.**
446, 2 mass units lower than the drug, and 2 mass units higher than the indole iminium ion metabolite. Its CID spectrum (data not shown) showed the base fragment ion exclusively at \( m/z \) 185. The CID mass spectrum of \( m/z \) 448 (\(^{37}\)Cl) of M6 also showed the fragment ion at \( m/z \) 185 as the almost exclusive product ion in the spectrum, suggesting that this fragment ion contains no Cl atoms and that the molecule had undergone ring closure and rearrangement to form a stable methyl-tetrahydro-pyrazino-indolylidene-like structure. Both the indole iminium ion metabolite and M6 (very likely to be the precursor of the indole iminium ion metabolite) were biosynthesized on a large scale, and the iminium ion metabolite was isolated and purified by HPLC. Treatment of the purified indole iminium ion metabolite with NaBH\(_4\) resulted in the disappearance of the indole iminium ion metabolite and the appearance of a new peak, which coeluted with M6 on HPLC (Fig. 9). A new HPLC method was developed with chromatographic separation for all human circulating metabolites (Fig. 10). The major human plasma metabolite M6 was coeluted with the biosynthetic M6 on HPLC and had an identical CID spectrum. Spiking a human plasma
sample with the purified radiolabeled biosynthetic M6 resulted in an increase of the radioactive response of M6 from the human plasma sample (data not shown). Both M6 and the chemically reduced indole iminium ion metabolite (after being treated with NaBH₄) have identical CID mass spectra (Fig. 11).

Figure 12 shows a comparison of the downfield region of the ¹H spectra of elzasonan (top), the above-identified indole iminium ion metabolite (middle), and metabolite M6 (bottom) from in vitro human incubations with rCYP3A4. It is clear by inspection that there are significant differences in the aromatic moiety of these compounds. The singlet at ~8 ppm in the elzasonan spectrum is missing in the two other spectra, indicating that the olefinic proton at the benzylidene moiety is not present in either metabolite. In addition, the singlet at ~5.7 ppm in the iminium ion spectrum is present in the metabolite M6 spectrum at ~5.6 ppm, indicating that metabolite M6 is a similar cyclized compound yielding an indole moiety. In addition, there is no peak at ~9 ppm in the M6 spectrum as there is in the iminium ion spectrum, indicating that the proton of the piperazine ring at position 3 is not adjacent to an iminium ion. These observations are consistent with the proposed cyclized structure. A complete synthetic pathway for M6 is described in the supplemental data and was used to confirm structure and test M6 for biological activity.

M6 accounted (average of six human subjects) for ~5.6% (sum of M5 and M6) of the administered dose in feces and ~65% of the total radioactivity in plasma and was not detected in urine. On the basis of the above data, M6 was identified as 4-(3,4-dichlorophenyl)-2-(2-methyl-1,2,3,4-tetrahydropyrazino[1,2-a]indol-10-yl)thiomorpholin-3-one.

In Vitro Covalent Binding of [¹⁴C]Elzasonan and Indole Iminium Ion Metabolite. Upon incubation of [¹⁴C]elzasonan with human liver microsomes, in the presence of NADPH covalent incorporation into microsomal protein was observed (mean = 191 ± 10 pmol/mg of protein in 20 min). In the absence of NADPH, little binding was observed. The addition of model nucleophiles, lysine and cyanide, did little to reduce the binding; however, the addition of reduced GSH resulted in a 52% decrease in binding. The binding was greatly reduced in the presence of ketoconazole, indicating that CYP3A activity was necessary to produce the intermediate that covalently binds to microsomal protein. The addition of cytosol, a source of aldehyde oxidase (an enzyme that converts alicyclic iminium ions to lactams) did not cause a reduction in binding. Binding was also observed in rat and dog liver microsomes, albeit to a lesser extent. The binding of iminium ions to nucleophilic groups represents a bond that is reversible in acid. Microsomes that were subjected to the same incubation conditions with [¹⁴C]elzasonan, but initially precipitated with weak perchloric acid, demonstrated a lower extent of covalent incorporation. The amount of covalent binding of radiolabeled material to microsomal protein under various conditions is listed in Table 4.

Biosynthesized [¹⁴C]elzasonan indole iminium ion was examined for metabolism dependent and independent of covalent incorporation into human liver microsomes. In the absence of NADPH, an average of 1% of the radiolabeled material was incorporated (1.9 pmol/mg of
protein) (Table 5). This binding was partially reduced in the presence of KCN or GSH. However, an enhancement of incorporation of radioactivity was observed in the presence of NADPH, suggesting that the iminium ion is further metabolized to other reactive intermediates.

**pH Titration Curve and Reaction of the Iminium Ion Metabolite with Cyanide and GSH.** The chemistry and reactivity of the iminium ion metabolite was examined. The equilibrium between iminium ions and carbinolamines will be dependent on the concen-
The iminium ion form is present, as observed through the absorbance maximum at 360 nm (Fig. 13A). As the pH was increased to 12.2, the absorbance decreased, and the inflection point of the titration was at approximately pH 10.5 (Fig. 13B). The iminium ion was shown to react with cyanide ion, as shown by the decrease in absorbance at 360 nm in Fig. 13C. However, attempts to isolate the α-cyanoamine and generate mass spectral data were unsuccessful. Mixing the iminium...
ion with increasing concentrations of GSH, up to 30 mM, did not cause any decrease in absorbance at 360 nm, indicating that the iminium ion metabolite does not form a stable adduct with GSH (data not shown).

Discussion

The present study describes the metabolism of elzasonan in human. A solution of [14C]elzasonan was administered orally to humans at a free base equivalent dose of 10 mg. Total recovery of the administered dose was 78.9 \( \pm \) 5.6%. Approximately 57.9 \( \pm \) 4.2 and 21.0 \( \pm \) 1.9% (mean \( \pm \) S.D.) of the administered radioactive dose was excreted in feces and urine, respectively (average of six human subjects).

Absorption of elzasonan was rapid as indicated by the rapid appearance of radioactivity in plasma after oral administration of [14C]elzasonan. The plasma concentrations of total radioactivity were always greater than those of unchanged drug, suggesting early formation of circulating metabolites. Elzasonan \( t_{1/2} \) was 32 h, and the plasma concentrations reached a peak of 60.4 ng/ml. On the basis of AUC(0–last), approximately 12% of the circulating radioactivity was attributable to unchanged drug. The balance, approximately 88%, of the plasma radioactivity was due to metabolites. Of the total radioactivity extracted from the plasma, the drug and metabolites (average of all human subjects) accounted for approximately 95%. The magnitude of the difference between concentrations of unchanged drug and radioactivity at all time points indicated that there are a considerable number of circulating

### TABLE 4

Covalent binding of [14C]elzasonan (10 \( \mu \)M) to liver microsomes under various conditions

Data are presented as means \( \pm \) S.D. Means represent three replicates.

<table>
<thead>
<tr>
<th>Microsomes</th>
<th>Condition</th>
<th>Covalent Binding pmol [14C]elzasonan Eq binding/microsomal protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>– NADPH</td>
<td>6.96 ( \pm ) 0.39</td>
</tr>
<tr>
<td></td>
<td>+ NADPH</td>
<td>191 ( \pm ) 10</td>
</tr>
<tr>
<td></td>
<td>+ NADPH, + GSH (5 mM)</td>
<td>910 ( \pm ) 10.6</td>
</tr>
<tr>
<td></td>
<td>+ NADPH, + KCN (0.1 mM)</td>
<td>165 ( \pm ) 29</td>
</tr>
<tr>
<td></td>
<td>+ NADPH, + lysine (1.0 mM)</td>
<td>185 ( \pm ) 16</td>
</tr>
<tr>
<td></td>
<td>+ NADPH, + ketoconazole (1.0 ( \mu )M)</td>
<td>27.5 ( \pm ) 2.7</td>
</tr>
<tr>
<td></td>
<td>+ NADPH, + cytosol (3 ( \mu )g/ml)</td>
<td>176 ( \pm ) 47</td>
</tr>
<tr>
<td></td>
<td>+ NADPH, acid precipitation</td>
<td>54.8 ( \pm ) 6.7</td>
</tr>
<tr>
<td>Rat</td>
<td>+ NADPH</td>
<td>33.6 ( \pm ) 7.8</td>
</tr>
<tr>
<td>Dog</td>
<td>+ NADPH</td>
<td>95.6 ( \pm ) 13.9</td>
</tr>
</tbody>
</table>

### TABLE 5

Covalent binding of [14C]elzasonan indole iminium ion (0.18 \( \mu \)M) to human liver microsomes under various conditions

Data are presented as means \( \pm \) S.D. Means represent three replicates.

<table>
<thead>
<tr>
<th>Condition</th>
<th>[14C]Iminium Ion-Related Radioactivity Bound pmol/mg microsomal protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.9 ( \pm ) 0.6</td>
</tr>
<tr>
<td>+ KCN (1.0 mM)</td>
<td>0.8 ( \pm ) 0.05</td>
</tr>
<tr>
<td>+ GSH (10 mM)</td>
<td>0.7 ( \pm ) 0</td>
</tr>
<tr>
<td>+ NADPH (1.3 mM)</td>
<td>4.0 ( \pm ) 0.2</td>
</tr>
</tbody>
</table>
metabolites. This finding corresponds well with the plasma radioactivity profiles. The high recovery of radioactivity attained from the extraction of plasma is most likely attributed to the absence of reactive iminium ion-related products.

Elzasonan was extensively metabolized and only a small amount of unchanged drug was found in urine and feces. Unchanged drug and the identified metabolites (average of six human subjects) accounted for approximately 85.2, 89.4, and 94.8% of the total radioactivity extracted from urine (0–120 h postdose), feces (0–336 h postdose), and plasma (0–96 h postdose), respectively.

The major component of drug-related materials in feces, urine, and plasma was unambiguously identified as the 5-hydroxyelzasonan metabolite (M3), the N-oxide metabolite of elzasonan (M5), and the novel cyclized indole metabolite of elzasonan (M6), respectively. There are no known circulating active metabolites of elzasonan.

A proposed scheme for the biotransformation pathways of elzasonan in human is shown in Fig. 14. Based on the structures of these metabolites, five major routes of metabolism were identified. The major routes included aromatic hydroxylation(s) of the benzyldiene
moiety, N-oxidation at the piperazine ring, indirect glucuronidation, N-demethylation and oxidation, and ring closure and rearrangement to form M6.

The formation of M6 is mediated by CYP3A4 and possibly initiated by single electron transfer from the nitrogen lone pair (N1) of the piperazine ring followed by deprotonation at the C-2 hydrogen adjacent to the N1 radical cation to form a neutral carbon radical intermediate (reaction a). Alternatively, hydrogen atom transfer from the C-2 hydrogen of the piperazine leads to the direct formation of the neutral carbon radical intermediate (reaction b). A second electron abstraction from the neutral carbon radical intermediate leads to the formation of the iminium ion intermediate. Direct formation of a carbinolamine without an intermediate iminium ion could take place and requires activated oxygen to react with the neutral carbon radical intermediate. The carbinolamine and iminium ion exist in a pH-dependent equilibrium. Under acidic conditions, it is likely that the iminium ion is the most probable form to exist. The two forms coexist in physiological pH (7.4) and weakly alkaline pH range, whereas carbinolamines exist in strong alkaline media. The nicotine iminium ion metabolite and its carbinol (5'-hydroxynicotine) metabolite have been reported to coexist in weakly alkaline media. In physiological pH (7.4) the carbinol form was estimated at approximately 25% (Brandange and Lindblom, 1979). Therefore, it is reasonably to assume that at physiological pH elzasonan carbinolamine and iminium ion equilibrium will shift toward the formation of the iminium ion metabolite. Ring closure and subsequent rearrangement (reaction c) followed by proton abstraction and valence tautomerism lead to the formation of M6. Further oxidation of M6 could result in the formation of the indole iminium ion metabolite. The formation of iminium ions as metabolic intermediates has been proposed to involve a cytochrome P450 catalyzed oxidation of the nitrogen atom by electron abstraction followed by a process of desaturation (Ortiz de Montellano, 1995; Vickers and Polsky, 2000). It has also been reported that alicyclic tertiary amine drugs such as phencyclidine (Ward et al., 1982; Hoag et al., 1987; Sayre et al., 1995; Driscoll et al., 2007), mianserin (Iverson et al., 2002; Argoti et al., 2005), and nicotine (Williams et al., 1990; Byrd et al., 1994; Carlson et al., 1995) undergo metabolism to form iminium ion metabolites. A possible mechanism for the formation of M6 and the indole iminium ion metabolite is shown in Fig. 15.

Low and variable recovery of radioactivity was observed after administration to human subjects (Table 1), which was not the case in rats or dogs (data on file, Pfizer, Inc.). In addition, in vitro metabolism studies had shown that elzasonan iminium ion was a quantitatively more important metabolite in human liver microsomes than in animal liver microsomes. Because iminium ion metabolites of drugs such as phencyclidine and nicotine have been shown to covalently bind to microsomal protein (Ward et al., 1982; Obach and Van Vunakis, 1988), an investigation of the potential for elzasonan to undergo metabolism-dependent covalent binding was undertaken in an attempt to address a hypothesis that the low recovery observed in the human excretion could be due to covalent sequestration of drug-related material. Upon incubation with liver microsomes and NADPH, [14C]elzasonan undergoes covalent incorporation into microsomal protein. This was determined by both extensive washing of added protein with organic solvent as well as extended dialysis of added protein (data not shown). It should be noted that although alicyclic iminium ion metabolites are commonly observed for cyclic tertiary amine drugs (Gorrrod and Aislatrner, 1994) and have been proposed as potentially reactive electrophiles (Oerton et al., 1985), there has been no direct evidence to link such metabolites with toxicity. For example, the metabolism-dependent covalent incorporation of mianserin, which forms an iminium ion, into liver microsomes does not appear to correlate with toxicity (Roberts et al., 1991).

The mechanism of nucleophile adduction to iminium ions has been explored for other drugs.Nicotine and phencyclidine are alicyclic tertiary amines that undergo metabolism to iminium ions, followed by further oxidation to the corresponding lactams by aldehyde oxidase. These two agents have also been shown to undergo metabolism-dependent incorporation into liver microsomes (Hoag et al., 1987; Shigenaga et al., 1988; Osawa and Coon, 1989), in which the presence of added nucleophiles, such as CN⁻ or GSH reduces the
extent of binding. However, for elzasonan, although GSH partially reduced the extent of incorporation, CN had no effect. For nicotine and phencyclidine, cyanide reacts at the α-position to form the cyanoamine (Hoag et al., 1987; Shigenaga et al., 1988). Cyanide adducts have been observed for several other amine drugs when incubated with liver microsomes (Gorrod et al., 1991). In addition, for nicotine iminium ion, the adduct formed between hydroxide ion and the iminium ion is also observed at pH greater than 7.5 (Brandange and Lindblom, 1979). However, for the elzasonan indole iminium ion metabolite, such an adduct (carbinolamine) was not formed until the pH was raised above 9.5 as assessed by an examination of the UV/VIS spectrum at different pH values, indicating that the elzasonan indole iminium ion metabolite may be less reactive to adduct formation than nicotine or phencyclidine iminium ions. This result can be rationalized by the conjugated nature of the iminium ion bond in the elzasonan metabolite (to the indole ring) in contrast with the unconjugated iminium ion bonds in nicotine and phencyclidine iminium ion, analogous to observations made for the dihydroisoquinolinium ion metabolite of nomifensine (Obach and Dalvie, 2006).

Although a cyano adduct of the elzasonan iminium ion metabolite could be observed as the UV absorption of the conjugated iminium ion bond decreased in the presence of potassium cyanide, such an adduct appeared to be relatively unstable because it could not be isolated. Furthermore, no reaction with GSH could be detected, because the UV absorption at 360 nm remained unchanged with an increase in the GSH concentration to 30 mM.

The iminium ion may not be the only binding species in the covalent incorporation of elzasonan-drug related material into liver microsomes. Although some covalent incorporation was observed for the biosynthesized indole iminium ion without metabolism, a greater amount of covalent incorporation was observed with further metabolism of the iminium ion (Table 5). This result was also observed for phencyclidine (Hoag et al., 1988). The structure of such an adduct has not been proven, but mechanisms have been proposed (Sayre et al., 1991, 1997).

It is tempting to hypothesize that the relatively low overall recovery of drug-related material in humans is due to the metabolic pathway that proceeds through M6 and ultimately yields the iminium ion metabolite, which could covalently bind to tissue nucleophiles. However, it is well established that low mass balance does not necessarily mean that a drug is demonstrating covalent binding in vivo. In an analysis of published human excretion studies on drugs known to undergo metabolism-dependent covalent binding, Roffey et al. (2007) found that drugs that can be bioactivated to reactive metabolites yield mass balance values in the same range as those of other drugs. Furthermore, it is known that drugs that have long plasma half-lives of total radioactivity and also those that are primarily excreted in the feces tend to have lower mass balance recoveries, and elzasonan...
possesses these properties. Thus, it cannot be concluded that all or part of the missing 21% of drug-related material is due to bioactivation to the iaminium ion metabolite.

In summary, the results of this study provide the first analysis of formation and excretion of metabolites of elzasonan in humans. Results were similar to those reported previously in preclinical species. Elzasonan is very rapidly eliminated in humans, mainly by phase I oxidative metabolism and subsequent phase II metabolic pathways. We have reported a full characterization of a novel circulating metabolite, resulting from oxidation at the piperazine ring and ring closure and subsequent rearrangement to form a pyrazino-indole structure.

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Metabolism, Pharmacokinetics and Excretion of the 5-HT_{1B} Receptor Antagonist Elzasonan in Humans

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[\textsuperscript{14}C]CP-448,187 was made by the following procedure

Step 1

[\textsuperscript{14}C]CP-678,396 (77.8 mCi, 57.5 mCi/mm mol, 1.35 mmol) was dissolved in 14.3 mL of dichloromethane and treated with pyridinium chlorochromate (475 mg, 2.20 mmol). After stirring at room temperature for 2 hours, the reaction was quenched with 1N aqueous sodium hydroxide, extracted with ethyl acetate and the organic layer was dried over anhydrous sodium sulfate and concentrated in vacuo. The resulting crude product residue was purified by silica gel chromatography, eluting with chloroform / ethanol (98:2) to yield [\textsuperscript{14}C]CP-423,622 (45.0 mCi, >98 % radiochemically pure). This material was dissolved and stored in ethyl acetate prior to being used in the next reaction.

Step 2

CP-457,431 (657 mg, 2.50 mmol) was dried from toluene azeotrope, dissolved in 7 mL of tetrahydrofuran (previously distilled from lithium aluminum hydride) and dried over magnesium sulfate. This solution was transferred to a 0 °C solution of sodium bis(trimethylsilyl)amide (1M in tetrahydrofuran, 0.83 mL, 0.83 mmol). After 6 minutes a solution of [\textsuperscript{14}C]CP-423,622 (45.0 mCi, 57.5 mCi/mm mol, 0.78 mmol) dissolved in 5 mL of tetrahydrofuran (previously distilled from lithium aluminum hydride) and dried over anhydrous magnesium sulfate was also transferred to the reaction. The cooling bath was removed and the reaction was allowed to warm to room temperature over 30 minutes. At that time HPLC indicated that the reaction was complete. The flask was
recooled to 0 °C and saturated aqueous ammonium chloride was added. The cold bath was removed and the reaction pH was adjusted to 5 with acetic acid. Much of the tetrahydrofuran was removed by rotary evaporation and the resulting solution pH was adjusted to 9 with aqueous sodium hydroxide. The remaining aqueous solution was extracted with ethyl acetate and the organic layer was dried over anhydrous sodium sulfate and concentrated in vacuo. The resulting residue was partitioned between ethyl acetate and water and solid sodium bisulfite was added. The biphasic solution was vigorously stirred for 1 hour, the layers were separated and the aqueous layer was extracted with additional ethyl acetate. The combined organic layers were washed with brine, dried over anhydrous sodium sulfate and concentrated in vacuo. The resulting crude product residue was purified by silica gel chromatography, eluting with ethyl acetate / methanol / triethylamine (98:1:1) to yield $[^{14}\text{C}]\text{CP}$-448,187 (32.4 mCi, >99 % radiochemically pure). This material was isolated in solid form by precipitation from hexane.

**M6 metabolite (CE-316,355) was made by the following procedure**

**Step 1**

Ice-cold DMF (150 mL) was purged with nitrogen gas and treated with sodium hydride (60% dispersion in mineral oil, 3.2 g, 80 mmol). A solution of ethyl indole-2-carboxylate (15 g, 79 mmol, 1 eq) in 50 mL of DMF was added dropwise at 0 °C. After the addition was complete the reaction was stirred for an additional 30 minutes before chloroacetonitrile (10 mL, 158 mmol, 2 eq) was added dropwise. After the addition was complete the reaction was heated at 65 °C for 1 hour. The reaction was then cooled to room temperature and quenched with excess ice-water, resulting in the formation of a light brown suspension. The suspension was filtered and the collected solids were rinsed with ice-water. The solids were dissolved in ethyl acetate, washed with brine, dried over anhydrous sodium sulphate and concentrated in vacuo to afford PF-00322235 (16.3 g, 72 mmol, 91 % yield) as an off-white solid.

**Step 2**

Ice-cold diethyl ether (125 mL) was purged with nitrogen gas and treated with lithium aluminium hydride (2 g, 53 mmol). Solid PF-00322235 (6 g, 26 mmol, 0.5 eq) was added while maintaining a positive nitrogen atmosphere. After the addition was complete the reaction was warmed to room temperature and then heated at reflux for 4 hours. The reaction was then cooled to room temperature and quenched by dropwise addition of 5% aqueous NaOH (2 mL) and then filtered through celite, rinsing with ethyl
Supplemental Data
DMD#34595

acetate. The filtrate was concentrated in vacuo to afford PF-00322320 (2.6 g, 15 mmol, 58% yield) as an off-white solid.

Step 3

PF-00322320 (5.7 g, 33 mmol, 1 eq) was dissolved in 175 mL of DMF. Solid K$_2$CO$_3$ (4.6 g, 33 mmol, 1 eq) was added followed by iodomethane (1.8 mL, 30 mmol, 0.9 eq) and the reaction was stirred at room temperature for 4 hours. The reaction was then diluted with excess brine and extracted with three portions of ethyl acetate. The combined organic layers were washed with brine, dried over anhydrous sodium sulfate and concentrated in vacuo. The resulting crude product residue was purified by silica gel flash column chromatography, eluting with 5% methanol in dichloromethane to afford PF-00217912 (4.4 g, 24 mmol, 73% yield) as a tan solid.

Step 4

CP-457,431 (1.0 g, 3.8 mmol, 1 eq) was dissolved in 10 mL of CH$_2$Cl$_2$. Sulfuryl chloride (0.5 g, 3.8 mmol, 1 eq) was added drop-wise and the resulting solution was stirred at room temperature for 15 minutes. The reaction solution was then concentrated in vacuo to afford PF-00420806 (1.1 g, 3.7 mmol, 97% yield) as an off-white solid.

Step 5

PF-00217912 (0.6 g, 3.2 mmol, 1 eq) and PF-00420806 (1.0 g, 3.4 mmol, 1.1 eq) were dissolved in 15 mL of CH$_2$Cl$_2$. Zinc triflate (0.2g, 0.5 mmol, 0.2 eq) and Hunig’s base (0.4 g, 3.4 mmol, 1.1 eq) were added and the reaction was stirred at room temperature under nitrogen for 48 hours. The reaction was then diluted with methanol and concentrated in vacuo. The resulting crude product residue was purified by silica gel
flash column chromatography, eluting with 20% acetone in chloroform to afford CE-316,355 (0.4 g, 0.9 mmol, 28% yield) as an off-white solid.